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(54) Title: FERROFLUID BASED ARRAYS

(57) Abstract: Ferrofluid-based magnetically generated microarrays are disclosed which are useful in high throughput screening assays for the identification, characterization and analysis of target entities in test specimens.

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**WO 01/51668 A1**

**FERROFLUID BASED ARRAYS**

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**CROSS REFERENCE TO RELATED PATENT APPLICATIONS**

10 This application claims priority under 35 U.S.C.  
section 119(e) to US Provisional Application Number  
60/175,828, filed January 13, 2000, the entire  
disclosure of which is incorporated by reference herein.

**15 FIELD OF THE INVENTION**

This invention relates to the fields of molecular  
biology and analytical methods. More specifically, the  
invention provides magnetically ordered arrays of  
ferrofluid particles on solid supports bearing at least  
20 one member of a specific binding pair having affinity  
for a target bioentity. Such arrays provide superior  
supports for assessing binding reactions including, but  
not limited to, those involving protein/protein,  
drug/protein, drug/oligonucleotide, complementary  
25 oligonucleotide hybridizations, complementary (cDNA,  
RNA) nucleic acids and protein/oligonucleotide for  
applications in screening, detection and analysis of  
target entities in test samples.

**30 BACKGROUND OF THE INVENTION**

Several publications and patents are referenced in  
this application in parentheses to more fully describe  
the state of the art to which this invention pertains.  
The disclosure of each of these publications and patents  
35 is incorporated by reference herein.

Many laboratory and clinical procedures employ

specific affinity-based interactions for isolating and analyzing target entities or species which are defined herein as substances or analytes in test samples of physiological, biological or chemical origin. Such  
5 affinity interactions are commonly employed in diagnostic testing and drug screening, or for the separation of a wide range of soluble and particulate target entities or analytes, particularly biological entities such as antigens, antibodies, proteins, nucleic  
10 acid sequences, oligonucleotides, cells, bacteria, viruses, and the like.

Various methods are available for analyzing or separating the above-mentioned target entities from test samples based upon complex formation between the target  
15 entity and specific binding substances which have affinity for the target. Selective separation of such complexes from unbound material may be accomplished gravitationally, e.g. by settling, or, alternatively, by centrifugation of small particles or beads having a  
20 substance with binding affinity for the target species attached thereto. Optionally and preferably, such particles or beads may be made magnetic to facilitate the separation of the bound fraction on the bead and the free fraction in the supernatant liquid. Limited  
25 applications of non-magnetic particles, (e.g., latex particles), in microarray applications, are discussed in the book entitled "DNA Microarrays, M. Schena, ed., pages 7-18, 1999.

Many different methods may be used to deposit  
30 biological entities onto microarray solid supports. These include capillary deposition, and photolithography/capillary deposition as described in US Patent 6,083,763. Alternatively, deposition of reactive

species on conventional microarrays may be accomplished using multiple ink jets. Small volumes of concentrated and thus viscous binding reagents are deposited to a defined area on either a non-reactive or chemically  
5 reactive planar surface using the jet methods. Such methods are also described in US Patent 6,110,426. This approach provides very low droplet volumes which minimizes reagents used and therefore associated costs.

Moreover, the printing process can be accelerated to  
10 thousands of droplets per second, thereby enabling a high throughput production capacity for reaction vessels. Reactive species may be applied to the array sequentially to provide a desired probe sequence or analyte (on-chip synthesis). Alternatively, complete  
15 pre-synthesized sequences or analytes may be applied to the chip in precisely controlled localized areas (off-chip synthesis). Such localized arrays are available from several companies, Erie Scientific Company, Portsmouth, NH being one example. The ink jet deposition  
20 methods can be further differentiated by the method by which the reagents are delivered. Such methods include, for example, piezoelectric capillary, piezoelectric cavity, thermal, acoustic, and continuous flow.

Application of binding reagents to chemically  
25 reactive surfaces results in the covalent and generally irreversible attachment of such reagents to the microarray surface. In contrast, non-reactive deposition of binding reagents often results in conformationally altered or denatured coatings with  
30 reduced analyte recognition and stability. Such non-reactive binding reagents also tend to wash off during incubations, thereby limiting the stringency of incubation conditions during hybridizations. The

denaturation problem can be minimized with single-point, oriented or site-specific attachment as is commonly done in reactive coating with mono-functionalized oligonucleotide probes or, for example, with biotin-  
5    avidin binding pairs. However, functionalization of the desired binding pair member is not always available. Additionally, when oligonucleotides are directly affixed to a solid support electrostatic interactions occur with the solid support which tend to interfere with efficient  
10    hybridization between the immobilized oligonucleotide and the target nucleic acid sequence. Clearly, improved methods which minimize conformational alterations of binding pair members during deposition on the surface of the microarray are highly desirable.

15       Magnetic particles are well known in the art, as is their use in immune and other specific affinity interactions. See, for example, US Patent No. 4,554,088 and Immunoassays for Clinical Chemistry, pp. 147-162, Hunter et al., eds., Churchill Livingstone, Edinburgh  
20    (1983). Generally, any material that facilitates magnetic or gravitational separation may be employed for this purpose. However, it has become clear that magnetic separation means are the method of choice.

      Magnetic particles can be classified on the basis  
25    of size as large (1.5 $\mu$ m to about 50 $\mu$ m), small (0.7 $\mu$ m to about 1.5 $\mu$ m), or colloidal (<200nm) which are herein interchangeably referred to as magnetic nanoparticles, ferrofluids, and colloidal or superparamagnetic particles. These nanoparticles consist of aggregates of  
30    smaller magnetite or maghematite crystals, where the latter is an oxidized form of magnetite.

      Magnetic nanoparticles of the type described herein are quite useful in analyses involving specific affinity

interactions, as they are conveniently coated with specific binding pair members to provide high surface areas and rapid reaction kinetics. Magnetic particles ranging from 0.7 $\mu$ m to 1.5 $\mu$ m have been described in the patent literature, including, by way of example, US Patent Nos. 3,970,518; 4,018,886; 4,230,685; 4,267,234; 4,452,773; 4,554,088; and 4,659,678. Certain of these particles are disclosed to be useful solid supports for immunological reagents.

10       The small magnetic particles mentioned herein generally fall into two broad categories. The first category includes particles that are permanently magnetizable, or ferromagnetic. The second category comprises particles that exhibit bulk magnetic behavior only when subjected to a magnetic field. The latter are referred to as magnetically responsive or superparamagnetic particles and consist of magnetite or hemagnetite crystals of about 30nm or less in diameter that can form the larger aggregates used in this invention.

20       Like the small magnetic particles mentioned above, larger magnetic particles (> 1.5 $\mu$ m to about 50 $\mu$ m), wherein the magnetite particles are dispersed or encapsulated in organic polymers, can also exhibit superparamagnetic behavior. Typical of such materials are those described by Ugelstad in US Patent No. 4,654,267 and manufactured by Dynal, (Oslo, Norway). The Ugelstad process involves the synthesis of polymer particles by microemulsion polymerization to form polymeric beads which are caused to swell and imbibe magnetite crystals. Magnetic particles in the same size range of 0.5 to 20 $\mu$ m can also be prepared by synthesizing the polymer particle in the presence of

dispersed magnetite crystals. Such syntheses result in the trapping of magnetite crystals in the polymer matrix, thus making the resultant beads magnetic. In both cases, the polymeric magnetic particles exhibit  
5 superparamagnetic behavior, which is manifested by the ability to disperse readily upon removal of the magnetic field. Unlike the magnetic colloidal nanoparticles previously referred to and discussed in further detail below, these large magnetic beads, as well as the larger  
10 magnetite particles, are readily separated with simple low-gradient magnetic separators because of the relatively high magnetic mass per particle. Hence separations with these larger particles can be done in conventional magnetic rack separators for test tubes or  
15 microtiter wells using gradients in the range of a few hundred gauss/cm to about 1.5 kilogauss/cm. Colloidal magnetic particles, below approximately 200nm in size, on the other hand, require substantially higher magnetic gradients, because of their diffusion energy, small  
20 magnetic mass per particle and Stokes drag, all of which must be overcome with high-gradient magnetic separators to effect efficient magnetic collection.

Another platform for detecting and measuring target analytes in a sample is described by PCT/US98/05911.  
25 These methods involve magnetically labeling the targets, and then pulling the target-magnetic particle complex to a collection surface that has the capability to bind the targets. This analytical method is based on two specific binding interactions to the target, the first  
30 by the particle, and the second by the collection surface. However, such methods are only suitable for use when the characteristic determinants of the targets are known to allow the specific binding interactions.

Furthermore, while methods are described for specific target binding in locations defined by the magnetic arrangement employed, there is no description of how to create specifically addressable locations (i.e.,  
5 creating an addressed array).

While magnetic particle assays and applications have been described and exemplified in the prior art in single-test or low-volume formats, the development of methods for performing such magnetic assays in high  
10 throughput screening modes is highly desirable. Such methods should facilitate the identification, characterization and analysis of biological or pharmaceutical target entities in test samples.

## 15 SUMMARY OF THE INVENTION

In accordance with the present invention, methods and apparatus for immobilizing and isolating target entities on solid supports for use in high throughput screening assays are disclosed. An exemplary method  
20 comprises immobilizing, and identifying a target entity in a test sample by means of magnetically ordered arrays in bounded compartments, by providing a solid support having a first member of a specific binding pair affixed thereto to which magnetic particles are added, the  
25 magnetic particles having affixed thereto a plurality of second members of a specific binding pair which have binding affinity for said first member of a specific binding pair. The magnetic particles are then focused in an array by magnetic means inside said compartments  
30 thereby essentially irreversibly immobilizing said magnetically responsive particles by interaction between said first binding pair member on solid support and said second binding pair member on said magnetic particles.



The immobilized particles in said compartments on said solid support are then contacted with a first nucleic acid sequence operably linked to said first member of a specific binding pair, thereby immobilizing said first  
5 nucleic acid sequence on said immobilized particles.

The immobilized particles bound by said first nucleic acid sequence are then contacted with a test sample suspected of containing a detectably labeled target entity having affinity for the first nucleic acid  
10 sequence. Binding between said first nucleic acid sequence and said target entity present in the test sample is detected and optionally quantified by measuring the detectable signal of said bound labeled target entity.

15 Suitable solid supports are selected from the group consisting of plastic surfaces, glass surfaces, silica surfaces, metal surfaces, membranes, microtiter plates, microtiter strips, and microtiter wells. The solid supports may contain bounded compartments overlying pin  
20 positions. In one embodiment, such compartments are formed by depositing hydrophobic lines on the solid support surface. Alternatively, the solid support may comprise a particle selected from the group consisting of polymeric microparticles, latex particles and  
25 inorganic microparticles ranging in size from about 0.5um to about 20um. Suitable magnetic particles for use in the present invention are selected from the group consisting of colloidal ferrofluids, magnetic nanoparticles, magnetic microparticles and ferrofluid-coated microparticles.  
30

In an alternative embodiment of the invention, a method is disclosed wherein a first nucleic acid sequence is operably linked to a biotin species and

placed in separate compartments on the array. The array is then contacted with a test sample containing a mixed population of detectably labeled nucleic acids, a subset of which are suspected to be complementary to a first  
5 immobilized nucleic acid. The immobilized first nucleic acid sequence and the test sample are subjected to conditions which permit hybridization between complementary nucleic acids, thereby permitting detection of a plurality of complementary target nucleic  
10 acids in said test sample. In an alternative embodiment, a plurality of nucleic acid sequences may be affixed to the array.

In a preferred embodiment of the invention, the first member of a specific binding pair is biotin or  
15 analogs thereof with comparable or lower affinity for avidin or avidin species, for example, desthiobiotin, and the second member of a specific binding pair is streptavidin or avidin species with comparable or lower affinity for biotin or biotin analogs, for example,  
20 avidin or nitro-avidin.

Suitable devices for measuring immobilized detectable labels are selected from the group consisting of fluorometers, spectrophotometers, scintillation counters, gravimetric biosensors, and surface plasmon  
25 resonance biosensors.

In yet a further embodiment of the invention, an apparatus for detecting a binding event between specific binding pair members is provided. An exemplary apparatus of the invention comprises means defining a  
30 magnetically ordered and responsive array on the surface of a non-magnetizable solid support, the array template bearing a plurality of magnetizable array pins in direct contact with said lower surface of said solid support.

The solid support being further divided into bounded compartments overlying the array pins, the array template being in further communication with a rare earth magnet or electromagnet, thereby generating a high magnetic gradient in proximity of the tips of said array pins. The gradient effectuates collection of magnetic particles in the magnetically responsive arrays. In a preferred embodiment, the array is a microarray with pin densities ranging between about 2 to about 100,000 per square inch, more preferably the pin densities range between about 4 and about 2000 per square inch.

The utility of the novel magnetic microarrays of this invention is further enhanced by the use of external magnetic gradients that permit simultaneous detection or screening of large number of target entities in a specimen sample. Alternatively multiple interactions of a single substance with a plurality of different specific binders, each indicative of a biological function may be identified and quantitated as exemplified by screening of drug candidates.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a graph showing the inhibition of binding of 5.5 $\mu$ m biotin beads to streptavidin-ferrofluid. The amounts of biotin-oligomer were increased to determine the optimum loading of biotin beads in relation to the maximum loading capacity of the streptavidin-ferrofluid particles.

Figures 2A-2C are a series of flow cytometry histograms depicting the increased fluorescence resulting from formation of specific binding complexes between specific binding pair members through a

hybridization reaction.

Figures 3A and 3B depict fluorescence images illustrating the effect of non-specific hybridization (Fig. 3A) and magnetically focused specific oligonucleotide hybridization (Fig. 3B) for a four-point macroarray of the present invention.

Figure 4A shows a magnified view (about 100x) of a microarray of streptavidin-ferrofluid irreversibly bound to a biotinylated support placed on top of a magnetizable microarray pin template, constructed of steel, bearing circular pins 0.05 mm in diameter, spaced 0.50mm apart, and mounted on top of a rare earth magnet. This microarray has about 2000 pins per square inch. Figure 4B is a schematic drawing of the reaction components shown in the microarray depicted in Fig. 4A.

Figure 5 is an apparatus for creating a homogenous array with a dispenser that delivers the particles, which are pulled to the collection surface. Such methods and apparatus may be used to create suitable arrays for assaying multiple samples for the presence of a single determinant.

25

Figure 6 is an apparatus for creating specifically addressed arrays, in which particles are delivered directly to the localized spot on the collection structure. Such methods and apparatus may be used to create arrays for assaying one sample for the presence of multiple determinants.

30

**DETAILED DESCRIPTION OF THE INVENTION**

This invention relates methods of production and multiple uses of ordered arrays of sub-micron sized ferrofluid particles on suitable planar or particulate solid supports for use as microarrays including so-called biochip configurations, although numerous others variations can be practiced. In one embodiment, the microarrays are formed by means of static magnetized pins which effect the formation of ferrofluid spots at locations on the microarray defined by the magnetic pins and bounded compartments corresponding to the pin locations. Such arrays may be used to advantage in numerous applications, including but not limited to gene expression analysis, hybridization assays for mutation detection, polymorphism analysis, genome mapping for the purpose of studying drug mechanisms, lead compound identification and optimization, combinatorial synthesis, array immunoassays, toxicity studies in pharmacodynamics and pharmacogenomics. Preferably all of the above identified reactions are performed using high throughput screening (HTS) methods.

One particularly promising technique for analyzing nucleic acid-containing biological samples uses a DNA-based microarray which generates a hybridization pattern representative of the characteristics of the DNA within the sample. Briefly, whereas conventional DNA microarrays included arrays of single stranded DNA fragments bound to a solid support, the methods and arrays of the present invention employ magnetic particles and magnetic fields to enhance the accuracy, reproducibility and detailed analysis of the binding reaction to be assessed. Each element within the array

includes few tens to millions of copies of identical single stranded nucleic acid sequences. Identical or different fragments of nucleic acids may be provided at each different element of the array. In other words, location (1,1) may contain a different single stranded fragment of nucleic acid than location (1,2) which may also differ from location (1,3) etc. Interactions between nucleic acid molecules may be assessed using chemiluminiscence, fluorescence, or electrical phenomenology.

A variety of devices for detecting such interactions are known. The most basic of these are purely chemical/enzymatic assays in which the presence or amount of analyte is detected by measuring or quantitating a detectable reaction product, such as gold immunoparticles. Specific binding pair interactions can also be detected and quantitated by radiolabel assays. In biosensor diagnostic devices, by contrast, the assay substrate and detector surface are integrated into a single device. One general type of biosensor employs an electrode surface in combination with current or impedance measuring elements for detecting a change in current or impedance in response to the presence of a ligand-receptor binding event, for example. This type of biosensor is disclosed, in U.S. Pat. No. 5,567,301.

Gravimetric biosensors employ a piezoelectric crystal to generate a surface acoustic wave whose frequency, wavelength and/or resonance state are sensitive to surface mass on the crystal surface. The shift in acoustic wave properties is therefore indicative of a change in surface mass, e.g., due to a specific binding pair interaction event. U.S. Pat. Nos. 5,478,756 and 4,789,804 describe gravimetric biosensors

of this type.

Biosensors based on surface plasmon resonance (SPR) effects have also been proposed, for example, in U.S. Pat. Nos. 5,485,277 and 5,492,840. These devices exploit  
5 the shift in SPR surface reflection angle that occurs with perturbations, e.g., binding events, at the SPR interface. Finally, a variety of biosensors that utilize changes in optical properties at a biosensor surface are known, e.g., U.S. Pat. No. 5,268,305. Biosensors have a  
10 number of potential advantages over binding assay systems having separate reaction substrates and reader devices. One important advantage is the ability to manufacture small-scale, but highly reproducible, biosensor units using microchip manufacturing methods,  
15 as described, for example, in U.S. Pat. Nos. 5,200,051 and 5,212,050. Another advantage is the potentially large number of different analyte detection regions that can be integrated into a single biosensor unit, allowing sensitive detection of several analytes with a very  
20 small amount of biological-fluid sample. Both of these advantages can lead to substantial cost-per-test savings.

Unlike other DNA array systems where the complementary nucleic acid sequence is synthesized *in*  
25 *situ* on the array biochip, the present invention allows for the direct deposition of the specific complementary oligonucleotide onto the solid support. In this embodiment, the oligonucleotides are operably linked to magnetic particles which are then magnetically focused  
30 onto the array as a single spot. As such, a simple unknown sample may be analyzed in a bulk fashion as a simple microarray composed of multiple magnetic particle spots, wherein each spot contains a different

complementary oligonucleotide sequence of interest. Similarly, multiple unknown samples may also be analyzed by specifically dispensing such samples onto individual spots.

5        In an exemplary method of the invention, wherein fluorescence imaging is employed, a target DNA sample to be analyzed is first labeled fluorescently. Each DNA sample is then denatured and hybridized to the microarray where complementary duplex formation is  
10 subsequently measured via fluorescence. Hybridization stringency is controlled such that each sequence target hybridizes only with its complementary nucleic acid sequence already affixed on the microarray. Nucleic acid sequences that are not complementary to any of the  
15 nucleic acids present on the microarray do not specifically hybridize to any of the sites of the microarray, and are washed away during subsequent wash steps.

      Thus, only those microarray locations containing  
20 probe sequences that hybridize with complementary sequences within the target DNA sample will receive the fluorescent molecules. Typically, a fluorescent light source is then applied to the microarray to generate a fluorescent image identifying which elements of the  
25 microarray bind to the patient DNA sample, for example, and those which do not. The image is then analyzed to determine which specific DNA fragments were contained within the original sample and to determine therefrom whether particular diseases, mutations or other  
30 conditions are present in the patient sample.

      For example, a particular element of the microarray may be exposed to sequences of DNA representative of a particular type of cancer. If that element of the array



fluoresces under fluorescent illumination, then the DNA of the sample contains the DNA sequence representative of that particular type of cancer. Hence, a conclusion can be drawn that the patient providing the sample  
5 either already has that particular type of cancer or is perhaps predisposed towards that cancer. As can be appreciated, by providing a wide variety of known DNA fragments on the microarray, the resulting fluorescent image can be analyzed to identify a wide range of  
10 conditions.

In general, there are two types of DNA microarrays: passive hybridization microarrays and active hybridization microarrays. Under passive hybridization, oligonucleotides characterizing the DNA sample are  
15 simply applied to the DNA microarray via solid-phase-probe and solution-phase-target where targets passively form complementary duplexes with the array probe. With active hybridization, the DNA array is configured to externally enhance the interaction between the fragments  
20 of the DNA samples and the fragments affixed to the microarray using, for example, magnetic techniques. It should be understood that, currently for any particular microarray, either the passive hybridization or the active hybridization steps, but not both, are typically  
25 employed. Referring first to passive hybridization, a DNA microarray chip is prefabricated with oligonucleotides of interest affixed or operably linked to solid-phase elements within the microarray via chemical or magnetic forces. The labeled sample target  
30 sequences are hybridized to the microarray. In one embodiment, the labeled sample target sequences that match any of the probe oligonucleotides affixed to the microarray passively hybridize while retaining their

fluorescent labels such that only those locations in the microarray having formed duplexes with the sample targets are fluorescent. It should be noted that high stringency hybridization conditions will ensure that  
5 duplexes form representing an exact probe sequence hybrid matches. When illuminated with fluorescent light, the exact matches fluoresces most effectively and the non-exact matches fluoresce considerably less or not at all.

10 The DNA microarray with the sample loaded thereon is placed within a fluidics station provided with chemicals to facilitate the hybridization reaction, i.e., the chemicals facilitate the hybridization of the sample sequences with corresponding complimentary probes  
15 within the microarray. The microarray is then illuminated under fluorescent light, perhaps generated using an ion-argon laser, and the resulting fluorescent pattern is digitized and recorded. Alternately, a photograph of the fluorescent pattern may be taken,  
20 developed, then scanned into a computer to provide a digital representation of the fluorescent pattern. In any case, the digitized pattern is processed using dedicated software programs that operate to focus the digital pattern and to subsequently quantify the pattern  
25 to yield a fluorescent intensity value for each array within the microarray pattern. The resulting focused array pattern is processed using additional software programs which compute an average intensity value at each array location and provides for necessary  
30 normalization, color compensation and scaling. Hence, following this step, a digitized fluorescent pattern has been produced identifying locations within the microarray wherein specific gene sequences from the DNA

sample have formed duplex hybrids.

As mentioned previously, the present invention is not limited to assessing interactions between complementary nucleic acid molecules. The methods  
5 disclosed herein may be utilized to assess binding interactions between any specific binding pair members.

The term "target entities" as used herein refers to a wide variety of soluble and particulate analytes or substances of biological or pharmaceutical interest.  
10 Examples of soluble target entities include hormones, proteins, peptides, lectins, drugs, chemical substances, oligonucleotides, nucleic acids (e.g., RNA and/or DNA) and particulate target entities which include biological particles such as cells, viruses, bacteria and the like.

15 The term "determinant", when used in reference to any of the foregoing target entities, refers to a portion or moiety of the target which is bound by a "binder" or any molecule which has specific binding affinity for the determinant. Such binders may or may  
20 not comprise one member of a specific binding pair. Determinants may consist of antigenic epitopes that are recognized by "antibody binders", nucleic acid sequences that are recognized by complementary nucleic acid  
25 "binders", or any molecule that is recognized by a specified binding partner. In fundamental terms, determinants are defined as molecular contact regions that are recognized in specific binding pair interactions. Preferred determinants are those that are unique to the target.

30 The expressions "specific binding pair" and "specific binding partner" are used interchangeably and refer to any two substances that selectively recognize and interact with each other, to the substantial

exclusion of other substances present in the sample medium. Specific binding partners comprising first and second binding pair members include, without limitation, antigens and antibodies, receptors and hormones, receptors and ligands, agonists and receptors, antagonists and receptors, lectins and carbohydrates, complemetary nucleic acid (RNA or DNA) and peptide-nucleic acid (PNA) sequences, Fc receptor and mouse IgG-protein A, biotin- or biotin analogs and avidin, biotin- or biotin analogs and streptavidin, metal chelating agents and metal ions, (i.e., EDTA or DTPA and  $\text{Fe}^{3+}$ ,  $\text{Cr}^{3+}$ , or  $\text{Ni}^{2+}$ ), enzymes and substrates and viruses and virus binding proteins. Various other specific binding pair combinations are contemplated for use in practicing the methods of this invention, and are readily apparent to those skilled in the art. Also contemplated for use in the invention are molecules such as peptides, drugs, nucleic acids, or analogs thereof which specifically recognize determinants with specificities similar to traditional antibodies.

The term "oligonucleotide," as used herein refers to primers and probes of the present invention, and is defined as a nucleic acid molecule comprised of two or more ribo- or deoxyribonucleotides, preferably more than three. The exact size of the oligonucleotide will depend on various factors and on the particular application and use of the oligonucleotide.

The term "probe" as used herein refers to an oligonucleotide, polynucleotide or nucleic acid, either RNA or DNA, whether occurring naturally as in a purified restriction enzyme digest or produced synthetically, which is capable of annealing with or specifically hybridizing to a nucleic acid with sequences

complementary to the probe. A probe may be either single-stranded or double-stranded. The exact length of the probe will depend upon many factors, including temperature, source of probe and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide probe typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides. The probes herein are selected to be "substantially" complementary to different strands of a particular target nucleic acid sequence. This means that the probes must be sufficiently complementary so as to be able to "specifically hybridize" or anneal with their respective target strands under a set of pre-determined conditions. Therefore, the probe sequence need not reflect the exact complementary sequence of the target. For example, a non-complementary nucleotide fragment may be attached to the 5' or 3' end of the probe, with the remainder of the probe sequence being complementary to the target strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the probe, provided that the probe sequence has sufficient complementarity with the sequence of the target nucleic acid to anneal therewith specifically. Probes may optionally contain a detectable label.

The term "specifically hybridize" refers to the association between two single-stranded nucleic acid molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a

single-stranded DNA or RNA molecule of the invention, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

5           The term "primer" as used herein refers to an oligonucleotide, either RNA or DNA, either single-stranded or double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed  
10 in the proper environment, is able to functionally act as an initiator of template-dependent nucleic acid synthesis. When presented with an appropriate nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme,  
15 suitable cofactors and conditions such as a suitable temperature and pH, the primer may be extended at its 3' terminus by the addition of nucleotides by the action of a polymerase or similar activity to yield an primer extension product. The primer may vary in length  
20 depending on the particular conditions and requirement of the application. For example, in diagnostic applications, the oligonucleotide primer is typically 15-25 or more nucleotides in length. The primer must be of sufficient complementarity to the desired template to  
25 prime the synthesis of the desired extension product, that is, to be able anneal with the desired template strand in a manner sufficient to provide the 3' hydroxyl moiety of the primer in appropriate juxtaposition for use in the initiation of synthesis by a polymerase or  
30 similar enzyme. It is not required that the primer sequence represent an exact complement of the desired template. For example, a non-complementary nucleotide sequence may be attached to the 5' end of an otherwise

complementary primer. Alternatively, non-complementary bases may be interspersed within the oligonucleotide primer sequence, provided that the primer sequence has sufficient complementarity with the sequence of the  
5 desired template strand to functionally provide a template-primer complex for the synthesis of the extension product. Primers may optionally comprise a detectable label.

The phrase "ink jet method" is used to refer  
10 methods suitable for deposition of desired reagents onto the solid support. The advantage of this deposition method is the ability to deposit very small amounts of reagent ( $<1\mu\text{l}$ ) in a precisely controlled location, as shown in Figure 6. Such deposition may be reversible or  
15 irreversible depending on the chemical reaction conditions employed.

The terms "operably linked" and "affixed thereto" are used interchangeably and herein refer to a covalent or non-covalent union of two molecules or a molecule and  
20 a solid support without impairment of either function.

The expression "detectably label" is used herein to refer to any substance whose detection or measurement, either directly or indirectly, by physical or magnetic means, is indicative of the presence of the target  
25 entity in the test sample. Representative examples of useful detectable labels, include, but are not limited to: optically detectable molecules or ions having light absorbance, fluorescence, reflectance, light scatter, phosphorescence, or luminescence properties; molecules  
30 or ions detectable by their radioactive properties; molecules or ions detectable by their nuclear magnetic resonance, magnetic remanence, or paramagnetic properties. Included among the group of molecules

single-stranded DNA or RNA molecule of the invention, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

5           The term "primer" as used herein refers to an oligonucleotide, either RNA or DNA, either single-stranded or double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed  
10 in the proper environment, is able to functionally act as an initiator of template-dependent nucleic acid synthesis. When presented with an appropriate nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme,  
15 suitable cofactors and conditions such as a suitable temperature and pH, the primer may be extended at its 3' terminus by the addition of nucleotides by the action of a polymerase or similar activity to yield an primer extension product. The primer may vary in length  
20 depending on the particular conditions and requirement of the application. For example, in diagnostic applications, the oligonucleotide primer is typically 15-25 or more nucleotides in length. The primer must be of sufficient complementarity to the desired template to  
25 prime the synthesis of the desired extension product, that is, to be able anneal with the desired template strand in a manner sufficient to provide the 3' hydroxyl moiety of the primer in appropriate juxtaposition for use in the initiation of synthesis by a polymerase or  
30 similar enzyme. It is not required that the primer sequence represent an exact complement of the desired template. For example, a non-complementary nucleotide sequence may be attached to the 5' end of an otherwise



molecules. In preferred embodiments, the molecular beacons are analyte specific and compete with or bind to the complementary oligonucleotide sequence as the target entity thereby providing a signal inversely proportional to the amount of target entity in the test solution. Hence, the reference to a detectable signal "quenched" refers to a nondetectable signal and "unquenched" to a detectable signal.

See Molecular Beacons: Probes that Fluoresce upon Hybridization, Sanjay Tyagi and Fred R. Kramer, Nature Biotechnology, volume 14 March, 1996.

Also included are non-detectable enzyme substrates which, by enzymatic action, are converted from non-detectable to detectable light absorbing or fluorescent molecules.

The phrase "to the substantial exclusion of" refers to the specificity of the binding reaction between a specific binder and its corresponding binding partner including a specific target entity in a specific binding pair. Specific binders have substantially selective binding affinity for their target entities, yet may also exhibit a detectable level of non-specific binding to other non-target entities or components in the test sample.

The term "species" as used herein collectively denotes the family of related substances consisting of isomers, analogs, and derivatives of a particular substance.

The term "microarray" or "biochip" refers to magnetically generated arrays on a planar surface that constitute a plurality of discrete reaction or incubation compartments identifiable by their locations or x-y coordinates on the array. Such arrays are

indirectly detectable based on light absorbance or fluorescence, are quenched fluorescent labels that do not fluoresce unless hybridized to the target sequence.

Suitable reagents for this purpose are "molecular  
5 beacons," as sold by RESEARCH GENETICS.

Molecular beacons, as used in the preferred  
embodiments, are probes that are useful for detecting  
specific nucleic acids in homogeneous solutions. Probes  
based on the molecular beacon principle are single-  
10 stranded nucleic acids molecules that possess a stem-  
and-loop structure. The loop portion of the molecule is  
a probe sequence that is complementary to a  
predetermined sequence in a target nucleic acid. The  
stem is formed by the annealing of two complementary arm  
15 sequences that are on either side of the probe sequence.

The arm sequences are unrelated to the target sequence.  
The fluorescent moiety is attached to the end of one  
arm and a non-fluorescent quenching moiety is attached  
to the end of the other arm. The stem keeps these two  
20 moieties in close proximity to each other, causing the  
fluorescence of the fluorophore to be quenched by  
fluorescence resonance energy transfer. As a result,  
the fluorophore is unable to fluoresce. When the probe  
encounters a target molecule, it forms a hybrid that is  
25 longer and more stable than the hybrid formed by the arm  
sequences. Thus, the probe undergoes a spontaneous  
conformational change that force the arm sequences apart  
and causes the fluorophore and quencher to move away  
from each other. This change in proximity allows the  
30 fluorophore to fluoresce when illuminated with the  
proper wavelength of light. These "Molecular Beacons"  
can only emit an increase in background fluorescent  
signal when they are hybridized to their target

0.09 to 0.15  $\mu\text{m}$  (90 to 150nm). Colloidal particles are characterized by stability and/or resistance to gravitational separation when left standing for extended periods. In addition to many other advantages,

5 particles in this size range rarely interfere with most detection techniques, for example, such particles do not interfere with microscopic analysis of target cells. Particles within the range of 90-150nm and having preferably between 5% to 99%, more preferably between

10 10% to 95%, and most preferably between 20% to 90% magnetic mass are contemplated for use in the present invention. Suitable magnetic particles are composed of a crystalline core of magnetite or maghematite surrounded by molecules which are coated or bonded, e.g. physically

15 absorbed or covalently attached, to the magnetic core and which confer stabilizing colloidal properties. The coating material should preferably be applied in an amount or thickness effective to fully shield the magnetite core from non-specific interactions with

20 biological macromolecules in the test sample. Such biological macromolecules may include single member of specific binding pairs, immunoglobulins, albumins, sialic acid residues on the surface of non-target cells, lectins, glycoproteins and other cell membrane components.

25 In addition, the material should contain as much magnetic mass per particle as possible. The size of the magnetic crystals comprising the particle core is sufficiently small so that they do not contain a complete magnetic domain. The nanoparticles are also

30 sufficiently small such that their Brownian energy exceeds their magnetic moment. Consequently, North Pole/South Pole alignment and subsequent mutual attraction/repulsion of these colloidal magnetic

suitable for use in assays for assessing specific binding characteristics between members of specific binding pairs.

The phrase "high throughput screening" is herein  
5 defined as any analytical system capable of processing a large number of test specimens for a plurality of target entities contained therein in a short time frame. Alternatively, high throughput screening may be utilized for characterizing the biological properties of a single  
10 entity by assessing its binding to a multiplicity of potential binding partners. For example, the Affymax Corporation has employed photolithographic methods to manufacture DNA chips or microarrays containing a large number of unique DNA probes. Immunoassay array panels  
15 bearing immobilized binding partners are disclosed in U.S. patents 4,591,570 and 4,829,010.

The phrase "conformational alteration" when used in connection with the deposition of binding species on the microarray includes minimal to total denaturation and  
20 further refers to undesirable changes in the tertiary structure of the binding reagent. Such alterations are particularly problematic in protein binding molecules which may lose up to 90% of their binding capacity on coating to a reactive or non-reactive surface due to  
25 multi-point attachment to the surface.

The expression "magnetized pins" or "pins" refers herein to formed protrusions mounted or machined on one surface of a magnetizable or non-magnetizable three-dimensional planar object.

30 The preferred magnetic particles for use in carrying out this invention are particles in the size range of 0.05 to 5um, more preferable in the range 0.05 to 0.5 um and most preferable in the colloidal range of

Scientific Company, Portsmouth, NH, in the formats of microarray glass plates and other substrates custom printed with hydrophobic ink in 96, 384, 1536 and higher patterns. The hydrophobic patterns for creating minute  
5 well compartments can be deposited with tight tolerances thereby making this technology highly compatible with and adaptable to the disclosed magnetic microarray concepts.

When utilizing such an array, ferrofluid particles,  
10 reagents and test solutions can be dispensed by means of conventional nanoliter or microliter delivery systems. Such systems include the aforementioned single or multi-channel high precision inkjet dispensers manufactured by DIE-MARK, San Diego, CA. The reagents may be deposited  
15 directly into the planar surface compartments, or into microtiter wells. As shown in Figures 5 and 6, precision ink jet dispensers are capable of delivering reagents to the entire sample, or to specific locations in the array. Specific binding pair members bearing  
20 characteristic determinants complementary to regions or sequences of interest on target species can be pre-bound to the ferrofluid particles before deposition or formation of the arrays by means of magnetized pins. In Figure 5, an apparatus is depicted which is suitable for  
25 assaying multiple samples for one determinant. The key feature of the apparatus and method distinguishing Figure 6 from Figure 5 is the ability to deposit and form ferrofluid spots containing ferrofluid particles of different specificities such that a multiplicity of  
30 target determinants can be detected from a single unknown sample.

In further embodiments of the invention, hybridization signals can further be amplified via any

particles occurs only minimally even in moderately strong magnetic fields, thereby contributing to their resistance to settling. Finally, the magnetic particles should be separable in high magnetic gradient external field separators that are characterized by generating a strong magnetic field in close proximity but external to the test sample.

Magnetic particles having the above-described properties can be prepared by modification of base materials described in U.S. Patents Nos. 4,795,698, 5,597,531, and 5,698,271.

There are a number of methods to create the arrays, which are more thoroughly described in the examples. However, each method relies on magnetically collecting particles that are capable of binding to the collection surface in precisely localized spots. The magnetic configuration that is employed will determine the physical layout of the array. The collection surface is coated with one member of a specific binding pair, while the magnetic particles are coated with the second member. Once the particles are drawn to the collection structure, they are bound to the surface.

In one embodiment of the invention, compartments were formed on a flat surface to contain up to 500uL from containment gaskets as described in Examples 3 and 4. Smaller surface compartments suitable for volumes in the 1 to 100uL range utilize hydrophobic lines or "fences" that can be readily constructed by conventional mechanical or lithographic line-forming means to prevent intermixing of the contents of adjacent array compartments.

Such patterned supports bearing compartments are commercially available from vendors such as Erie

porous supports or into preformed wells as in microtiter plates containing 96, 384, 1536, and higher number of discrete spots, for example, as about 2000 spots per square inch as depicted in Fig. 4. 5) Ferrofluids allow the use of magnetic field detectors to non-destructively measure the mass and thus the number of particles deposited, thereby creating an internal standard allowing normalization of the detected signal. 6) Reversibly releasing and recapturing the three-dimensional magnetic microarray spots by magnetic means increases the kinetics of affinity reactions by allowing the ferrofluid particles to locally mix in suspension in the absence of a magnetic field; ferrofluid particles return to the original spot configuration upon application of the magnetic field before substantial diffusion of the magnetic particles occurs.

One method for producing the microarray of the invention employs magnetic focusing by means of magnetized pins that give rise to "points" of labeled ferrofluid particles in the appropriate magnetic field in a planar pattern. This method permits each location to be uniquely defined, addressable and thus traceable to a binding interaction with a particular target entity.

"Magnetized pins" form protrusions mounted or machined onto one surface of a magnetizable or non-magnetizable three-dimensional planar object, as shown in Figure 5. Such pins (54) being non-magnetic, will become magnetizable when exposed to a magnetic field from a magnet applied distal to the pins. The magnet (55), either an electromagnet or a removable strong permanent magnet of the rare earth type, induces strong magnetic gradients in proximity of the tips of the pins

conventional nucleic acid amplification method such as polymerase chain reaction (PCR), ligation chain reaction (LCR), in vitro RNA polymerase/promoter driven amplification, (with variants such as aRNA, TMA, 3SR etc.), and rolling circle amplification technology (RCAT). Furthermore, any selective compartment signal can now be amplified via standard signal amplification methods. Possible hybridization buffers include any and all suitable reactions mixes including such reagents as volume excluders, hybridizing rate enhancers and sensitivity enhancers (i.e. dextran sulfate, polyethylene glycol, phenol, etc.)

Unique benefits are provided when ferrofluids are employed as a substrate for the creation of indexed microarrays as disclosed in this invention. Such advantages include those listed below: 1) Colloidal ferrofluid can be dispensed like ordinary liquids using conventional pumps or inkjet delivery systems since the particle concentrations and the resulting viscosities are low. 2) The dispensed ferrofluid particles may be focused out of solution in numerous shapes exemplified by very tight points, groups of points, or lines, allowing the use of significantly dilute solutions and low reagent concentrations, as well as very precise control of the actual array shape and size. 3) Ferrofluids may be deposited either reversibly in a three-dimensional pattern using non-binding supports which are held magnetically, or irreversibly deposited using binding supports that provide a thin layer of bound ferrofluids in a two-dimensional pattern (substantially a monolayer of particles) that remains firmly attached even after removal of the magnetic field. 4) Ferrofluids may be dispensed onto flat non-



label, which corresponds to the amount of a target molecule present. The detection limit for target entities, for example, in a fluorimetric immunoassay, is further enhanced by the ability to focus the signal in a very small area. In contrast, conventional fluorimetry focuses the light beam on only a fractional volume of the incubation mixture and thus does not allow detection of all fluorophores present.

An alternative method uses detectably labeled small non-magnetic microspheres, e.g. 0.5-10um in size, also bearing one or more encapsulated fluors, further bearing a surface that is covalently bound to a first member of a specific binding pair. The magnetic particle bears the second member of the specific binding pair and is capable of forming the bound pair, immobilizing the particle. The resultant bound layer on the magnetic particle renders the microspheres magnetically responsive and also permits attachment of additional binding partners or labels. Such magnetic microspheres can also be magnetically manipulated and focused into point or linear arrays. After binding of specific binding pair members, one of which contains a detectable label, the microparticles can be analyzed on the microarrays with a device such as a scanning laser spectrophotometer in a high throughput screening mode. As an internal standard of the magnetic collection efficiency, the mass or number of the magnetic microparticles at a given array site may also be determined by means of a magnetic particle detector, for example, the MAR II, described by Quantum Design (WO 99/27369).

An exemplary analysis involves the following step sequence. The binding partner for a target is attached

that collect the magnetic ferrofluid particles (52) on the solid surface (53) proximal to the pins, thereby forming an array pattern. Once collected, the streptavidin-bearing ferrofluid particles in the array are either immobilized reversibly (as three-dimensional thick layers) or irreversibly (as two-dimensional monolayers after wash-off of non-bonded ferrofluids) depending on the absence or presence of covalently attached specific binding pair member on the surface. An exemplary ink jet dispenser (51) is also shown.

Figure 6 exemplifies the synthesis or formation of a single ferrofluid spot. A derivatized thin solid support (64) containing covalently attached ligands (63) having an affinity for a complementary moiety on the ferrofluid particles (62), is placed atop a magnetized pin (65). The tip (61) of a dispenser is shown that is positioned extremely closely above the magnetized pin and the arrows depict the very rapid migration of the ferrofluid particles, that are ejected from the tip, towards the magnetized pin. The focusing of ferrofluid particles in this fashion allows for the formation of very small spots whose diameter is limited by only the diameter of the pin that is used. In this way, ferrofluid spots ranging in diameter from sub-micron sizes to micron sizes can easily be formed. The distinguishing feature of Figure 6 from Figure 5 is the ability to deposit and form ferrofluid spots containing ferrofluid particles of different specificities such that a multitude of targets can be detected from a single unknown sample.

This will allow determination of not only the presence or absence of the target molecule, but also its relative concentration. This can be accomplished because the signal of the spot will be known, and can be compared to the relative intensity of a detectable

analysis of the spots allows correlation of relative fluorescence measurements with magnetic field measurements as an internal reference standard permitting more accurate determination of the target  
5 species by normalization or correction of the measured signal for variations in the amount of collected ferrofluid particles.

The following examples are provided to illustrate various embodiments of the invention. They are not  
10 intended to limit the invention in any way.

#### EXAMPLE 1

15       **USE OF 5.5 $\mu$ m DIAMETER MAGNETIZED BIOTINYLATED  
POLYSTYRENE BEADS AS A SOLID-SUPPORT TO EFFECT  
HYBRIDIZATIONS**

This example illustrates the preparation of a  
20 ferrofluid reagent for use as a substrate in DNA hybridization. A biotinylated oligonucleotide was added in increments to streptavidin ferrofluid followed by biotinylated polystyrene microsphere beads. The magnetically collected microspheres were visualized and  
25 counted by bright field microscopy.

Increasing amounts (0 to 4423 picomoles) of a 26-  
mer 5'-biotinylated oligomer (mw=8684D), that encodes  
for the protein  $\beta$ -actin (OPERON TECHNOLOGIES INC.,  
Alameda, CA), were mixed and incubated with a fixed  
30 amount (50 $\mu$ g iron) of Streptavidin ferrofluid (Cat.#  
F3107; IMMUNICON CORP., Huntingdon Valley, PA). Next, 1  
 $\times 10^6$  particles of 5.5 $\mu$ m biotinylated microspheres (Cat.#

to the surface of the magnetic particles. This complex is mixed with the sample to promote the capture of the target onto the magnetic particles. The magnetic particle-target complex is then focused and collected  
5 onto the solid support collection surface for signal and analysis.

Ferrofluids provide superior substrates for molecular interactions because reaction kinetics of the dispersed ferrofluid with the target species or with the  
10 labeling species can be substantially increased in the reversible array mode compared to the irreversible array mode or the solid phase mode in conventional microarray assays. This reversible array mode is possible when the arrayed particles are not bound to surface and can thus  
15 be manipulated by removing and restoring the magnetic field. As the holding force is removed, the ferrofluid particles will diffuse off the surface, permitting more efficient interaction of the reacting species. However, before substantial diffusion occurs, the magnetic force  
20 is reapplied, and the spot is reformed in its original position.

Besides optical detection or quantitation of the target species on the array by conventional means, measurement of the deposited magnetic mass permitting  
25 calculation of the number of magnetic particles can also be done non-destructively. With the recent advances in miniaturized high-sensitivity remnant or residual magnetic field detection devices (e.g. by means of magnetic assay readers, MAR II, as manufactured by  
30 Quantum Design, San Diego, CA), such high-density ferrofluid arrays are easily localizable and quantifiable down to levels of 100 to 10,000 ferrofluid particles per spot. This non-destructive method for

streptavidin for binding additional biotinylated species.

To a partially saturated magnetic biotin-bead-streptavidin complex (about 50% saturated with biotin-oligo-26-mer)), an oligomer complementary to the  $\beta$ -actin sequence containing a quenched fluorophore, which is non-fluorescent when not hybridized (Molecular Beacon from RESEARCH GENETICS, Huntsville, AL) was added at about 10% of the 26-mer level (Fig. 2C). After a sufficient period of incubation, each mixture, including appropriate controls, were analyzed on a FACSCalibur Flow Cytometer (BECTON DICKINSON, San Jose, CA) for fluorescent events, where such detectable events depict successful hybridization. Corresponding controls without microbeads (Fig. 2A) and without biotin-oligomer (Fig. 2B) were also run. Figure 2 shows the three histograms. The hybridized sample (Fig. 2C) showed significant amounts of fluorescence events (>99%) indicating that ferrofluid immobilized biotin-oligomers undergo efficient hybridization reactions.

The aforementioned magnetic microbeads can be successfully utilized to form arrays by immobilizing single or multiple populations of magnetized microbeads in an ordered fashion on a planar surface by means of magnetizable focusing pins mounted in a non-magnetic planar support beneath and in contact with the planar surface. This is accomplished by magnetically attracting and focusing the magnetic particles in array points with a movable magnet or an electromagnet situated below the magnetizable focusing pins. Precise pipetting of the relatively non-viscous ferrofluid suspensions permits consistent loading of the spots. One can deposit the ferrofluid beads reversibly or irreversibly on a solid

CP10N ; BANGS LABORATORIES INC., Fishers, IN) were added to each mixture and left to incubate with mixing at room temperature for 48 hours. Then, with the aid of a Microwell Magnetic Protein Separator (Cat.# GS4108 ;  
5 IMMUNICON CORP., Huntingdon Valley, PA), all of the above mixtures were allowed to magnetically separate for 10 minutes, were washed while in the magnetic field, and finally were resuspended into TE buffer (TRIS / EDTA / MgCl<sub>2</sub>, pH 7.0). Each magnetic bead mixture was then  
10 counted in a hemocytometer to quantify the number of collected magnetized beads. The results show a maximum binding capacity of about 13 units of biotin oligomer per 50ug streptavidin ferrofluid.

The results of this experiment are shown in Figure  
15 1. The data indicate that it is possible to successfully synthesize magnetized streptavidin beads which, after conjugation to a biotinylated oligonucleotide, retain residual biotin binding sites for further immobilization onto a biotinylated substrate  
20 or solid phase.

The method can be extended to include multiple oligonucleotides of different complementarities or specificities to permit the analysis of a plurality of target analytes.

25

## EXAMPLE 2

### QUANTIFICATION OF HYBRIDIZATION REACTIONS ON BEADS

30 Hybridization reactions were carried out with several of the magnetized biotin-oligomer-ferrofluid bead mixtures prepared in Example 1 close to the midpoint, since these bead mixtures have available

**TABLE 1**

HYBRIDIZATION CONDITION	MEAN FLUORESCENCE INTENSITY	% MAGNETIC FLUORESCENCE EVENT
CONTROL (just beads)	2	0
ExpressHyB <sup>1</sup> @ 68° C	214	95.3
6x SSC <sup>2</sup> @ 68° C	348	99.7
6x SSC/0.5% SDS @ 68° C	210	99.1
TE/MgCl <sub>2</sub> @ 68° C	338	99.9
1x HyB @ 55° C	293	98.8
ExpressHyB @ 37° C	376	100
6x SSC @ 37° C	283	99.8
TE/MgCl <sub>2</sub> @ 37° C	299	98.8

5   <sup>1</sup> ExpressHyB (Clontech, Palo Alto, CA)-proprietary formulation.

<sup>2</sup> SSC - a 6x solution has 0.9M sodium chloride and 0.09M sodium citrate.

**EXAMPLE 4**

10       **PREPARATION AND USE OF A GLASS SUBSTRATE BASED  
          FERROFLUID ARRAY**

     This procedure describes preparation of  
     irreversibly bound streptavidin-ferrofluid spots on a  
15   biotinylated glass substrate, but any one of numerous

types of organic or inorganic solid phases such as polystyrene, polycarbonate, mylar, silicon wafers and the like can be similarly used. If excess unbound ferrofluid is washed away, the resultant layer of streptavidin-ferrofluid is irreversibly bound to the surface and does not require magnetic separations. The spot arrays in planar or multi-well formats are stable and can be preformed for used in high throughput screening assays for target species applied as ultra thin layers to reduce diffusion times.

In this Example, glass cover-slips (24mm x 40mm) of #1 thickness (Cat. # C7931; SIGMA CHEMICAL COMPANY, St. Louis, MO) were acid-etched first by treatment with concentrated hydrochloric acid for 20 minutes, washed with de-ionized water, followed by treatment with concentrated nitric acid for 20 minutes, washed again with de-ionized water, soaked briefly in methanol, and then oven dried at 90°C for 10 minutes. Next, the etched cover-slips were treated with a 10% ethanolic solution of N-2-aminoethyl)-3-aminopropyltrimethoxysilane (Cat.# 80379; PIERCE, Rockford, IL) for 16 hours at room temperature. The cover-slips were then heated at 90°C for 1 hour, allowed to cool, washed sequentially with de-ionized water, ethanol and de-ionized water and finally dried at 90°C for 1 hour. These aminosilanized cover-slips were biotinylated with a 10mg/ml dimethylsulfoxide solution of biotin-succinimide ester (Cat.# S-1582; MOLECULAR PROBES INC., Eugene, OR) for 4 hours at room temperature on an orbital rotator. After washing copiously with both de-ionized water, 20mM HEPES, and buffer of 0.15M sodium chloride, 0.1% sodium azide, pH7.5, the cover-slips were treated with a solution of 5% bovine serum albumin, phosphate buffered saline, 0.1%



sodium azide for 4 hours at room temperature. After further washing with HEPES buffer, the coated cover-slips were left to dry at room temperature.

Next, a plastic gasket (25mm x 20mm x 2.5mm) forming a shallow incubation chamber was placed atop one of the cover-slips and the assembly was allowed to rest on a four-point array consisting of four 13mm long nails mounted in a non-magnetizable breadboard with the pointed nail-ends touching the underside of the cover-slip. This assembly in turn was placed on top of a 13mm x 13mm x 50mm neodymium/iron/boron permanent magnet with the nail heads touching the magnet. The pointed nail-ends encompassed a circle of about 0.3mm diameter.

The gasket-enclosed incubation chamber was filled with 400µl of a 10µg/ml Streptavidin ferrofluid solution and left for 10 minutes to form a ferrofluid spot array on the cover-slip surface. Then the cover-slip was removed from the magnetized nail-bed, washed copiously with de-ionized water and HEPES buffer to remove loosely bound ferrofluid and allowed to dry at room temperature for several days before use.

To one of these cover-slips fitted with a plastic gasket, 400 µl of a 10µM 26-mer-biotin-oligomer solution in TE buffer was added and left to incubate for 2 hours at room temperature. After washing the cover-slip with de-ionized water, HEPES buffer and drying at room temperature, 400µl of a 1µM solution of the molecular beacon was added. After a period of 20 minutes, the cover-slip was placed into a FOTODYNE-UV box equipped with a camera, to monitor and record any fluorescence emission from the hybridization reaction. A negative control, where the biotin-oligomer incubation is

omitted, was also processed on a separate cover-slip that contained the ferrofluid 'spots'.

Figure 3B shows the results of this experiment and it is readily apparent that efficient hybridization had taken place in the four ferrofluid 'spots' where the biotin-oligomer was immobilized and resulted in bright white 'spots'. Less fluorescence is seen on the negative control cover-slip (Fig. 3A) and in the areas adjacent to the ferrofluid spots.

#### EXAMPLE 5

##### CONSTRUCTION AND USE OF MICROARRAY PIN TEMPLATES.

In Figure 4A, streptavidin-ferrofluid was irreversibly focused and immobilized onto a biotin-coated glass surface defined by the pin locations with minimal binding to adjacent non-magnetic areas. A schematic drawing of the reaction components is shown in Figure 4B.

The microarray pin template was constructed from a 25mm x 25mm x 25mm block of a magnetizable stainless steel cube one surface of which was machined to produce a pin array of about 45 x 45 pins, each pin having a diameter of 0.050mm with a separation of 0.50mm, to provide a pin pattern with about 2000 spots per square inch. As in Example 3, the pin template was sandwiched between the biotinylated cover-slip resting on the pins and a rare earth magnet (13mm x 13mm x 50mm) in contact with the underside of the cube distal to the pin pattern.

Streptavidin-ferrofluid was placed on a gasket-enclosed biotinylated cover-slip and allowed to incubate for 10 min to focus the ferrofluid in discrete spots.

The cover slip was removed and the excess loosely bound ferrofluid was rinsed off as in Example 3. After drying, a section of the microarray was magnified about 100X under a bright field microscope and photographed (Fig. 4A). A sharply defined microarray pattern with minimal binding to unfocused adjacent areas was obtained.

Alternatively, one can dispense preformed biotinylated complexes of streptavidin bearing biotinylated binding partners or detectable biotinylated labels all of which can be simultaneously accommodated on the same particle since the particles have a relatively high binding capacity as shown in Fig. 1.

The gaskets enclosures used in these Examples hold about 500uL solution. Lower volumes in the 1-100uL range can be contained in square compartments bounded by hydrophobic borders or "fences" deposited mechanically or lithographically to prevent intermixing of materials in adjacent array sites.

Similarly, larger magnetic microparticles prepared by coating conventional biotinylated microparticles with streptavidin-ferrofluid, for example in the range 0.5 to 10um, can be used to deposit a magnetic layer on the microparticles and thus make them subject to magnetic manipulation.

25

#### EXAMPLE 6

#### IMMOBILIZING AND IDENTIFYING A SINGLE TARGET ENTITY IN A MICROARRAY COMPRISING MULTIPLE BINDERS SUSPECTED OF HAVING BINDING AFFINITY THEREFOR

A similar but reversibly bound pattern can also be generated on non-binding surfaces or inside microtiter wells of 96, 384, 1536 or larger arrays, all of which can be manipulated by magnetic means. Conventional inkjet or other dispensing means may be

used to deposit different binders or magnetic binder complexes into separate wells to provide effective means for high throughput screening of test samples for a plurality of target species with affinity for different binders as in gene profiling. Or, conversely, one can test a single target entity with a plurality of different binders as in drug profiling and toxicity studies. Hybridization studies with RNA and DNA substrates, templates or amplicons can also be performed by the methods of this invention.

Numerous further variations encompassing the principles, methodologies and processes disclosed in this invention can be contemplated.

The exemplary embodiments have been primarily described with reference to diagrams illustrating pertinent features of the embodiments. Each method step may also represent a hardware or software component for performing the corresponding step. It should be appreciated that not all components or method steps of a complete implementation of a practical system are necessarily illustrated or described in detail. Rather, only those components or method steps necessary for a thorough understanding of the invention have been illustrated and described in detail. Actual implementations may utilize more steps or components or fewer steps or components. Thus, while certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.

What is claimed is:

1. A method for immobilizing, and identifying a target entity in a test sample by means of magnetically ordered arrays in bounded compartments, said method comprising:
  - a) providing a solid support having a first member of a specific binding pair affixed thereto;
  - b) adding magnetic particles having affixed thereto a plurality of second members of a specific binding pair having binding affinity for said first member of a specific binding pair;
  - c) focusing said magnetic particles in an array by magnetic means inside said compartments thereby essentially irreversibly immobilizing said magnetically responsive particles by interaction between said first binding pair member on solid support and said second binding pair member on said magnetic particles;
  - d) contacting said immobilized particles in said compartments on said solid support with a first nucleic acid sequence operably linked to said first member of a specific binding pair, thereby immobilizing said first nucleic acid sequence on said immobilized particles;
  - e) contacting said immobilized particles and said first nucleic acid sequence with a test sample suspected of containing a target entity having affinity for said first nucleic acid sequence, said target entity being detectably labeled; and
  - f) detecting binding between said first nucleic acid sequence and said target entity present in the test sample by measuring the detectable signal of said bound labeled target entity.

2. A method as claimed in claim 1, wherein said solid support is a support selected from the group consisting of plastic surfaces, glass surfaces, silica surfaces, metal surfaces, membranes, microtiter plates, microtiter strips, and microtiter wells.

3. A method as claimed in claim 1, wherein said solid support bears bounded compartments overlying pin positions and wherein said compartments are formed by depositing hydrophobic lines on said solid support surface.

4. A method as claimed in claim 1, wherein said solid support is a particle selected from the group consisting of polymeric microparticles, latex particles and inorganic microparticles ranging in size from about 0.5um to about 20um.

5. A method as claimed in claim 4, wherein said particles range in size from about 0.5um to about 5 um.

6. A method as claimed in claim 1, wherein said magnetic particles are selected from the group consisting of colloidal ferrofluids, magnetic nanoparticles, magnetic microparticles and ferrofluid-coated microparticles.

7. A method as claimed in claim 1, wherein said first member of a specific binding pair is a biotin species and said second member of a specific binding pair is a streptavidin species.

8. A method as claimed in claim 1, further comprising the step of quantifying the amount of target bioentity present in said test sample.

5        9. A method as claimed in claim 1, wherein said first nucleic acid sequence is operably linked to a biotin species and placed in separate compartments on the array, and said test sample contains a mixed population of detectably labeled nucleic acids, a subset  
10 of which are suspected to be complementary to said first nucleic acid, subjecting said first nucleic acid sequence and said test sample to conditions which permit hybridization between complementary nucleic acids, thereby permitting detection of a plurality of  
15 complementary target nucleic acids in said test sample.

10. A method as claimed in claim 1, wherein said detectable label is selected from the group consisting of fluorescent labels, radioactive labels,  
20 chemiluminescent labels and magnetic labels.

11. A method as claimed in claim 1, wherein said detectable label is detected using a device selected from the group consisting of fluorometers,  
25 spectrophotometers, scintillation counters, gravimetric biosensors, and surface plasmon resonance biosensors.

12. An apparatus for detecting a binding event between specific binding pair members comprising:  
30        means defining a magnetically ordered and responsive array on the surface of a non-magnetizable solid support, said array template bearing a plurality of magnetizable array pins in direct contact with said

lower surface of said solid support; said solid support being divided into bounded compartments overlying said array pins; and said array template being in further communication with a rare earth magnet or electromagnet, thereby generating a high magnetic gradient in proximity of the tips of said array pins, said gradient effectuating collection of magnetic particles in said magnetically responsive arrays.

10       13. An apparatus as claimed in claim 12, wherein said compartments are bounded by hydrophobic lines generated by mechanical or lithographic means.

15       14. An apparatus as claimed in claim 12, wherein said array is a microarray with pin densities ranging between about 2 to about 100,000 per square inch.

20       15. An apparatus as claimed in claim 14, wherein said pin densities range between about 4 and about 2000 per square inch.

25       16. An apparatus as claimed in claim 12, wherein said array template is constructed of magnetizable pins mounted in a non-magnetizable template.

30       17. A apparatus as claimed in claim 12, wherein said pins are generated on one surface of a magnetizable array template by a means selected from the group consisting of mechanical or lithographic means.

18. An apparatus as claimed in claim 12, wherein said magnetically responsive arrays are manipulated by altering the magnetic fields in proximity of said pins.



19. An apparatus as claimed in claim 12, wherein  
the collected magnetic particles are analyzed by optical  
5 means. .

20. An apparatus as claimed in claim 12, wherein  
the collected magnetic particles are analyzed by  
measuring a magnetic signal.

Figure 1

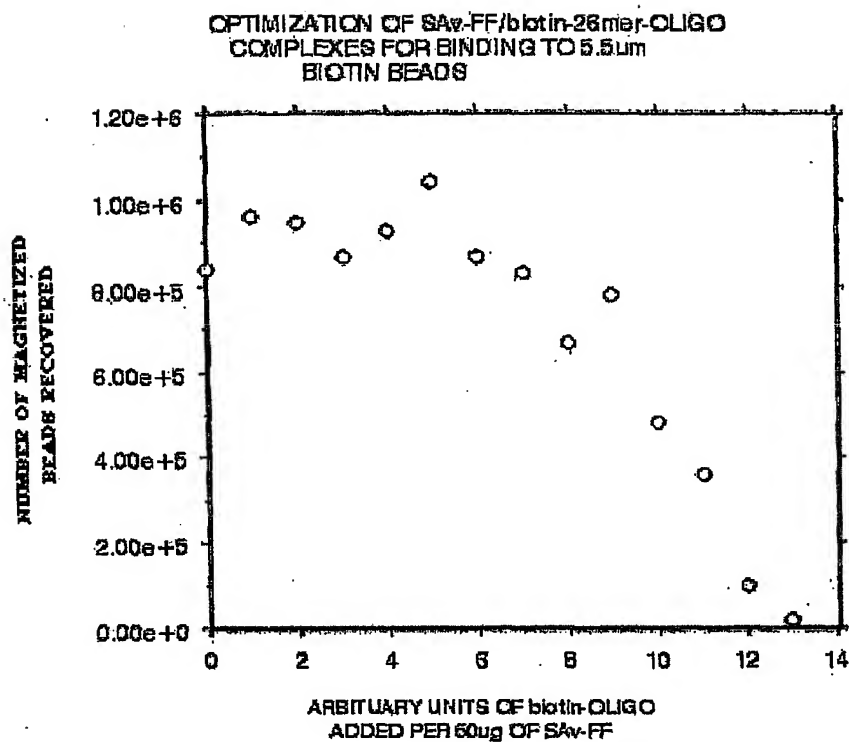


Figure 2A

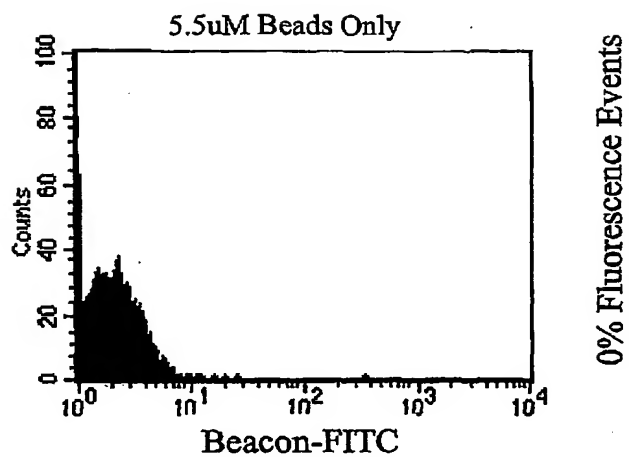


Figure 2B

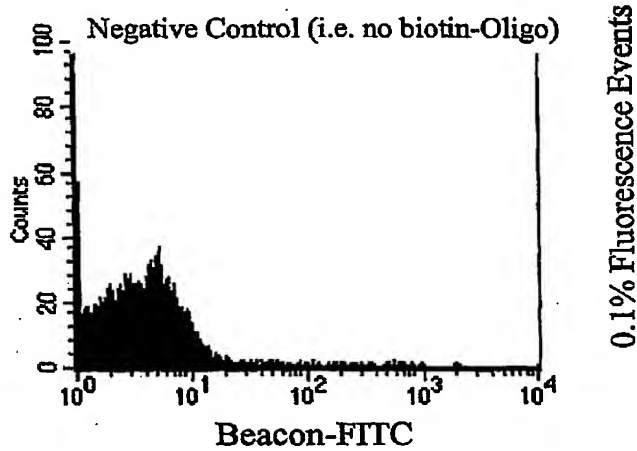


Figure 2C

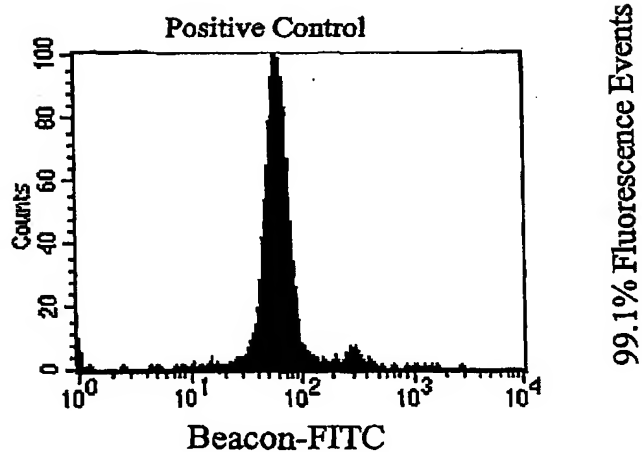


Figure 3A: Negative Control

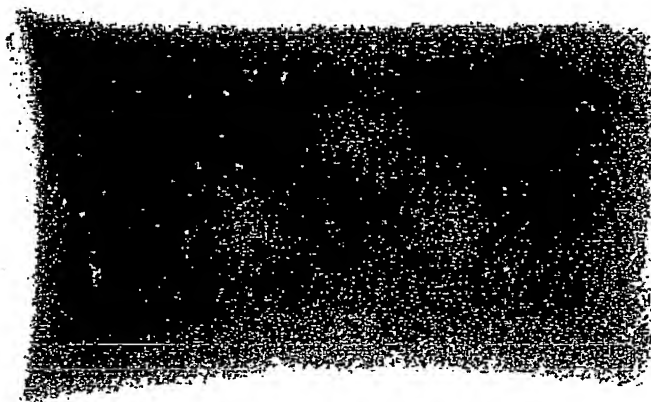


Figure 3B: Positive Control

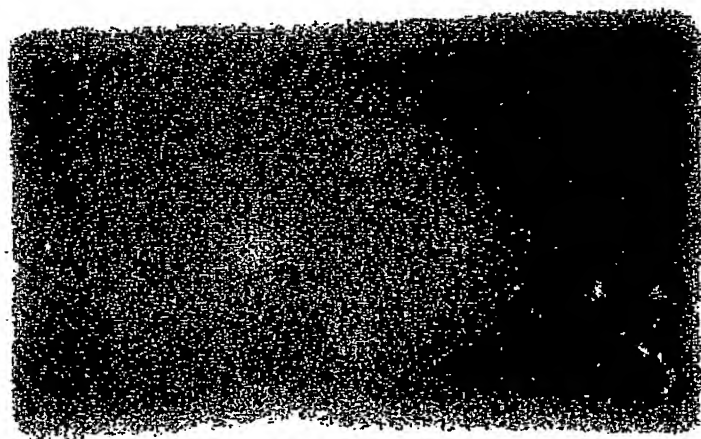


Figure 4A

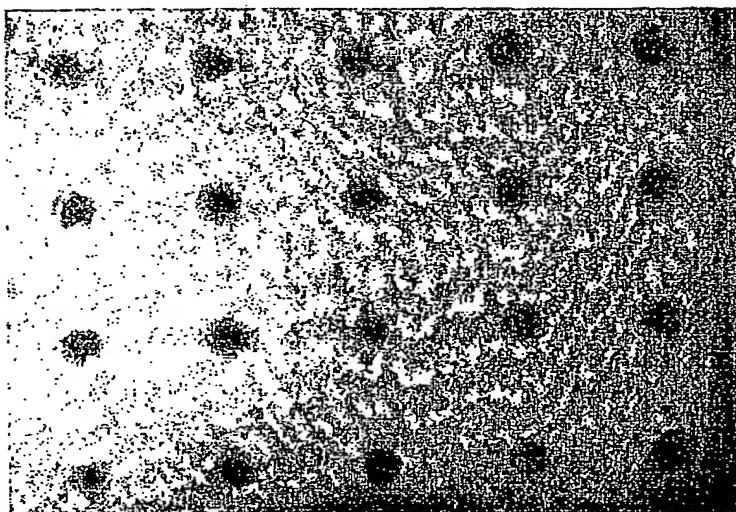


Figure 4B

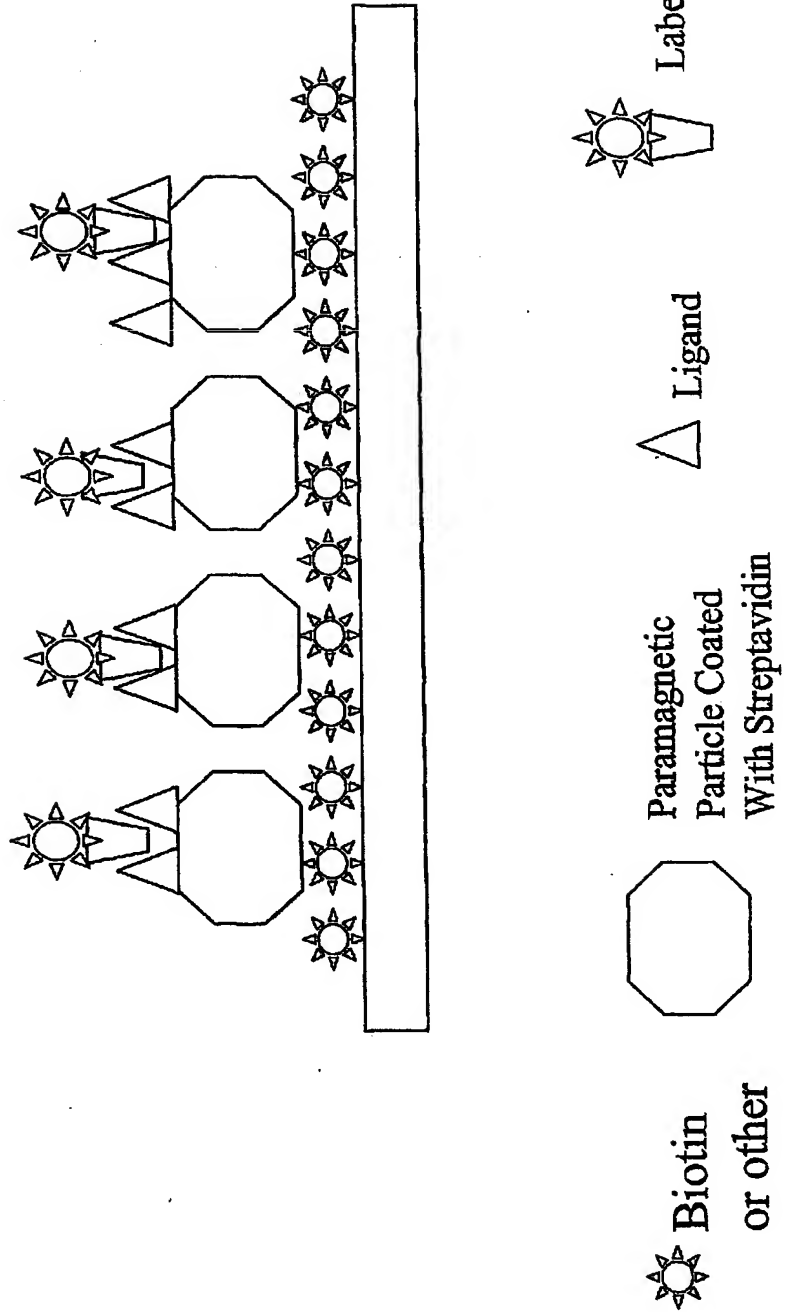


Figure 5

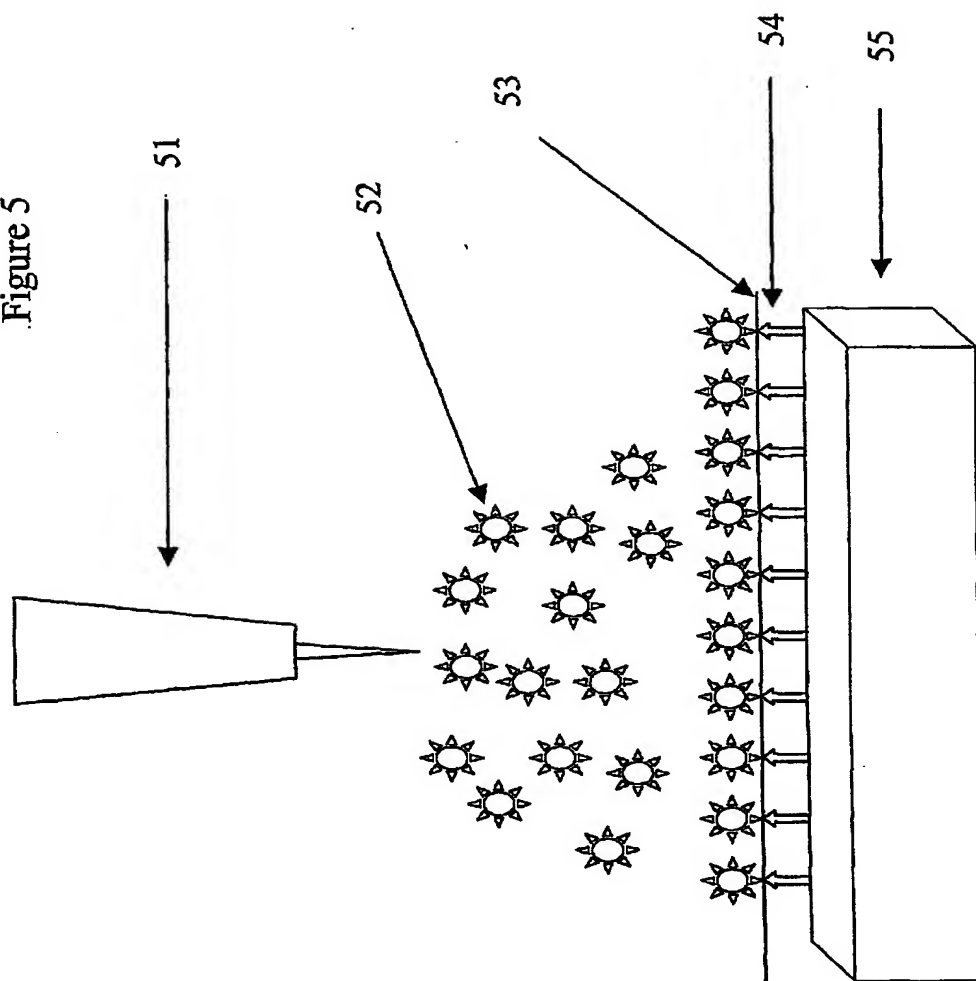
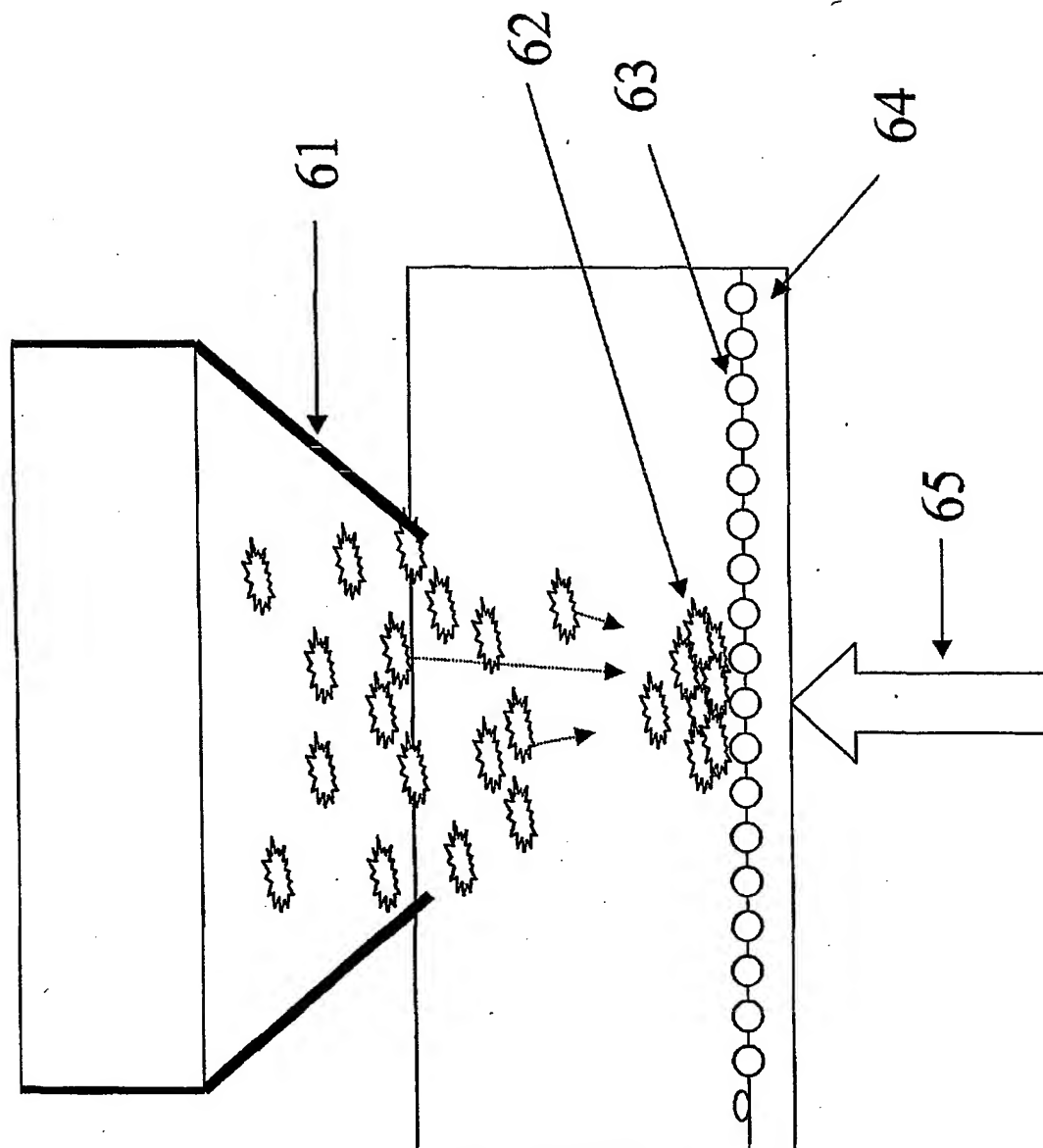


Figure 6





## INTERNATIONAL SEARCH REPORT

 International Classification No.  
 PCT/US01/01378

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q 1/68; C12P 19/34, C12M 1/34

US CL : 435/6, 91.2, 287.2

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.2, 183, 287.2; 436/94; 530/350; 536/23.1, 24.3, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,108,933 A (LIBERTI et al.) 28 April 1992, see entire document.	1-20
Y	US 5,200,084 A (LIBERTI et al.) 06 April 1993, see entire document.	1-20
Y	US 5466574 A (LIBERTI et al.) 14 November 1995, see entire document.	1-20
Y	US 5,610,287 A (NIKIFOROV et al.) 11 March 1997, see column 22, first full paragraph.	1-20
Y	US 5,698,400 A (COTTON et al.) 16 December 1997, see column 19, line 14, bridging to column 20, line 4.	1-20
Y, P	US 6,030,782 A (ANDERSON et al.) 29 February 2000, see column 8, last paragraph.	1-20

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

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Date of the actual completion of the international search

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Date of mailing of the international search report

30 MAR 2001

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/01378

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	US 6,100,040 A (RAMBERG) 08 August 2000, see column 4, line 60, bridging to column 5, line 9.	1-20
Y, P	US 6,110,684 A (KEMPER et al.) 29 August 2000, see column 2, third paragraph.	1-20
Y, P	US 6,121,023 A (ROMANO et al.) 19 September 2000, see column 6, lines 42-49.	1-20

# INTERNATIONAL SEARCH REPORT

Int. application No.  
PCT/US01/01378

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

EAST

Search Terms: magnet\$, probe, primer dna, nucleic acid, array, hydrophobic, surface tension, biotin, avidin, streptavidin, detect\$

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